resorcinol method. The high molecular weight fraction was found to contain 0.6 mg BSA/mg Pn14.

Example 10: Preparation of Amino-oxy-BSA – Neisseria PsC Conjugate

Neiss PsC was oxidized to create terminal aldehyde as generally described in Jennings & Lugowski *J. Imm.* 127:1011 (1981). SEC HPLC indicated the molecular weight of the PsC was significantly reduced.

After overnight conjugation of PsC and BSA-AO, analysis was conducted via SEC HPLC Superose 6 0.5 ml/min. The conjugate was fractionated on a 1x60cm S200HR column, equilibrated 10 mM sodium acetate, 150 mM NaCl, 2 mM EDTA, pH 5. It was determined by both SEC analysis and gel filtration that most of BSA was conjugated. The high molecular weight peak was analyzed for protein and carbohydrate and determined to contain 0.2 mg BSA/mg PsC.

Example 11: Preparation of amino-oxy-BSA – Neisseria PsA conjugate

This example illustrates the preparation of a Neisseria PsA-BSA conjugate by way of functionalizing the protein with an amino-oxy group.

Neiss PsA was terminally reduced to an alditol with $NaBH_4$ and then oxidized to create terminal aldehyde as generally described in Jennings & Lugowski *J. Imm.* 127:1011 (1981).

Neisseria PsA was solubilized in water at 20 mg/ml for 15 min. To 1 ml of the solubilized polysaccharide, 10 mg of sodium borohydride was added. The pH was maintained to about 8-9. After 1 hour, 100 µl of 1 M NaAc was added, and the pH was adjusted to 5. The reduced PsA was desalted on a 1x15 cm G10 column, equilibrated with saline, and the void volume fraction concentrated with an Amicon Ultra 4 (10 kDa cutoff device) to about 1 ml. 20 mg of solid sodium periodate was added, along with 100 µl 1 M sodium acetate at pH 5. After a 15 minute oxidation in the dark at room temperature, the reaction was quenched by the addition of a drop of glycerol and then desalted on 1x15cm G10 column

equilibrated with 10 mM NaAC, 150 mM NaCl and 2 mM EDTA, pH 5 (acetate buffer). The void volume was pooled and found to be positive in the BCA assay, indicating the presence of reducing sugar. The material was diafiltered and concentrated with an Ultra 4 device into acetate buffer.

Both the amino-oxy BSA and the PsA(red/ox) were examined by SEC HPLC. The molecular weight of the PsA was markedly reduced by the reduction/oxidation process.

Conjugation

In the conjugation step, 150 µl amino-oxy-BSA at 6 mg/ml was combined with 50 µl PsA (red/ox) and 25 µl 1 M NaAc at pH 5.

After an overnight incubation in the dark at 4° C, the conjugate was analyzed by SEC HPLC (Superose6, saline, 0.5 ml/min). It was seen that the PsA contributed very little absorbance and the AO-BSA increased in molecular weight on conjugation.

The conjugate was fractionated on a 1x60cm S200HR gel filtration column and the high molecular weight fraction assayed for protein and PsA and was found to contain 0.4 mg BSA/mg PsA.

Conclusion: The reduction/oxidation method works well to create aldehydes that can be linked to amino-oxy-protein. PsA was probably hydrolyzed during the $NaBH_4$ step, which is at elevated pH.

Example 12: Preparation of PRP(ox)-BSA-AO Conjugate

1. Oxidation of PRP Hib

22.7 mg PRP Hib was made up at 10 mg/ml in water, and combined with 100 µl 1 M NaAc and 46 µl 0.5 M sodium periodate. The reaction proceeded in the dark and on ice for 15 minutes, and was then quenched with 50% glycerol. The reaction mixture was diafiltered into water with an Amicon Ultra 4 (10 kDa cutoff) device, 4 x 4ml, final volume was approximately 1 ml. A resorcinol assay

was conducted at 10 mg/ml. The sample was positive in the BCA assay, indicating the presence of aldehyde.

2. Conjugation amino-oxy BSA

AO-S-BSA was provided at 15 mg/ml. 667 µl BSA-S-AO 10 mg was combined with 100 µl 1 M sodium acetate, at pH 5, and approximately 1 ml PRP(ox), and the reaction was permitted to proceed overnight in the dark. It was then quenched by the addition of 50 µl 0.25 M amino-oxy acetate.

3. Assay by SEC Superose6 prep grade HR10/30, equilibrated PBS 0.5 ml/min, OD 220.

Here, 0.5 ml conjugate was fractionated on 1x60 cm S200HR, and equilibrated PBS. All fractions eluted before BSA, indicating higher MW.

Fraction	BSA/mg Hib PRP (mg)
9	0.7
10	0.6
11	0.4
12	0.5

Example 13: Preparation of BSA-Pn14 Conjugate via Oxidation of Glycidic Acid

This example illustrates a protocol whereby glycidic acid was added to amines on BSA using carbodiimide. The glycidic acid on the protein was then oxidized and reacted with amino-oxy-Pn14.

1. BSA-Glycidic acid

Monomeric BSA and glycidic acid (obtained from Fluka Chemical) were combined to a final concentration in water of 12.5 mg/ml and 28 mg/ml, respectively. The pH was adjusted to about 5 and 220 µl of 100 mg/ml EDC in water was added. The pH is kept at about 5 for approximately 1.5 hours, and the

reaction was quenched by the addition of .025 ml 1 M sodium acetate at pH 5. The reaction mixture is then dialyzed against saline at 4°C overnight.

II. Oxidation of BSA-glycidic acid

100 µl of 1 M sodium acetate at pH 5 was added to 1 ml of BSA-glycidic acid (7.8 mg/ml), followed by 25 µl of 0.5 M sodium periodate in water. After 10 minutes in the dark, glycerol was added to quench the reaction and excess reagent removed using an Amicon Ultra centrifugal device with a 30 kDa cutoff. The final volume was about 400 ul.

100 µl of the oxidized BSA-glycidic acid was combined with 250 µl of amino-oxy Pn14 at 9.3 mg/ml along with 50 µl 1 M sodium acetate at pH 5. An aliquot was evaluated by SEC HPLC (Superose 6 0.5 ml/min, PBS). After an overnight reaction, another aliquot was assayed in the same way. It was seen that a significant portion of the absorbance eluted at the void volume (~15 minutes), indicating that the protein was linked to the high molecular weight Pn14.

Following gel filtration on an S400HR column (Pharmacia), the high molecular weight fraction was determined to contain 0.3 mg BSA/mg Pn14. This ratio is similar to that determined from the percentage of conjugated high molecular weight protein in the above chromatogram.

Thus, the method of linking glycidic acid to protein using carbodiimide provides a way to create aldehydes on proteins that can be subsequently linked to amino-oxy groups.

Example 14: Use of Amino-Oxy Chemistry to link BSA to Dextran

This example illustrates the coupling of an oligosaccharide via its reducing end to amino-oxy derivatized protein.

T40 dextran was made up at 100 mg/ml in water. The number of reducing ends was estimated using the BCA assay with glucose as the standard. It was

found that there were 3.5 mM reducing ends/100 mg/ml T40 dextran, so the average molecular weight was taken to be approximately 28,000 kDa

5 mg amino-oxy BSA containing ~8 amino-oxy/BSA was combined with 2 ratios of T40 dextran at pH 5.

(A) 830 µl BSA-AO 15.3 mg/ml was combined with 620 µl T40 dextran at 100 mg/ml and 100 µl 1 M NaAc at pH 5.

(B) 830 µl BSA-AO 15.3 mg/ml was combined with 3.1 ml T40 dextran at 100 mg/ml and 500 µl 1 M NaAc at pH 5.

Solutions were reacted at room temperature in the dark for 1 week, and then assayed by SEC HPLC.

. Both conjugates eluted much earlier than BSA-AO, indicating that their molecular weight has increased. Conjugates were then fractionated by anion ion exchange (IEX). Consistent with the SEC profile, the higher the molecular weight, the lower ionic strength the conjugate eluted. IEX elution fractions were analyzed for the ratio of carbohydrate to protein and plotted on both a weight and mole ratio (using 28 kDa MW for the T40 dextran)

The peak fraction from each IEX elution was analyzed by SEC HPLC (Superose 6 1 ml/min). The absence of monomeric BSA and the increasing MW for high vs. low ratio T40dex/BSA conjugates. SDS PAGE confirmed the high molecular weight nature of the conjugate IEX eluants.

Example 15: Use of Amino-Oxy Chemistry to Link Oligosaccharide and Protein

This example demonstrates the use of amino-oxy chemistry to link an oligosaccharide indirectly via its reducing end to a protein. A general description of the protocol is as follows. The reducing end of T40 dextran (~40 kDa MW) was reacted with the amino-oxy group of amino-oxy acetate to create a dextran with a single carboxyl group on one end. This carboxy group was then converted to an

amine by reaction with ethylenediamine and carbodiimide. The amine-tipped dextran was then thiolated and reacted with maleimide-derivatized BSA, to create a conjugate consisting of a protein with "threads" of carbohydrate extending from it.

I. Addition of amino-oxy acetate to the reducing end of dextran

850 mg of T40 dextran (Pharmacia) was solubilized in 850 µl of water overnight at room temperature.

235 mg of amino-oxy acetate was solubilized in a mixture of 850 µl DMSO and 500 µl 1 M sodium acetate, pH 5 and combined with the T40 dextran solution. An additional 500 µl of DMSO was added to make the solution approximately 50% DMSO. After an incubation at about 68°C for about 6 hours, the solution was extensively dialyzed against water. The product was dextran containing a single carboxyl group on its reducing end.

1.8 g of ethylenediamine 2HCl was added to the solution (approximately 22 ml) and the pH adjusted to approximately 5 with 1 N NaOH. 220 mg of EDC (1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride) was added and the pH maintained at about 5 for 3 hours. The reaction was then quenched by the addition of 1 M sodium acetate, pH 5, dialyzed against saline, and concentrated using an Amicon Ultra 15™ (10 kDa cutoff). It was then further dialyzed against saline and then against water.

The product was assayed for amines using TNBS and for carbohydrate using the resorcinol assay. It was determined that there were approximately 0.45 amines per 40,000 kDa of dextran. This product was dextran containing a single amine group on its reducing end and was termed NH_2-AOAc-T40 dextran. Using the resorcinol assay, the solution was determined to have a concentration of about 119 mg/ml dextran. T40 dextran consists of a distribution of molecular

weights, which makes it difficult to determine the actual degree of substitution of the reducing ends of the polymers.

II. Thiolated dextran and maleimide- BSA

Maleimide-derivatized BSA was prepared as follows: GMBS (40 µl of a 0.1 M stock in NMP) was added to a solution of 200 µl of monomeric BSA (42.2 mg/ml), 50 µl 0.75 M HEPES, 5 mM EDTA at pH 7.3, and 100 µl water. After a 2 hour reaction, the pH was reduced by the addition of 100 µl 1 M sodium acetate, at pH 5. The solution was desalted using an Amicon Ultra 4™ (30 kDa cutoff) ultrafiltration device and 10 mM NaAc, 0.15 M NaCl, 5 mM EDTA, pH 5.

The NH_2-AOAc-T40 dextran was thiolated using SPDP as follows: 0.5 ml of the NH_2-AOAc-T40 dextran was combined with 100 µl of 1 M HEPES, pH 8 and 100 µl of 0.1 M SPDP were added. After approximately 2 hours, 50 µl of 0.1 M EDTA pH 5 was added, followed by 100 µl of 1 M sodium acetate, pH 5 and 50 µl of 0.5 M dithiothreitol in water. After a 1 hour incubation, the solution was dialyzed into sodium acetate buffer overnight at 4°C.

The thiol tipped T40 dextran and the maleimide derivatized BSA were combined (a small aliquot of the BSA-maleimide was saved for analysis). After an overnight reaction, one half the mixture (about 1 ml) was fractionated by gel filtration using a 1x60 cm S-400HR column, equilibrated with saline. For comparison, a mixture of 100 µl BSA monomer (42.2 mg/ml), 300 µl T40 dextran AOAc, and 0.5 ml saline was similarly fractionated on the same gel filtration column. Fractions (about 1 ml) were analyzed for protein by absorbance at 280 nm and for dextran using the resorcinol assay.

With reference to Figures 5A-D, it is evident that the protein and dextran are eluting earlier from the column when in the T40 dextran AOAc-thiol-maleimide BSA conjugate than when the components are mixed. This indicates that a

conjugate of higher molecular weight has been formed. Furthermore, the ratio of dextran to protein increased.

The column fractions were further analyzed by SDS PAGE, with the results provided in Figure 6. From left to right, MW marker, conjugate fractions 18, 20,22,24,26, mixture fractions # 24,26,28, 30, unfractionated conjugate, starting BSA-maleimide.

It is evident that the unfractionated conjugate contains only a small proportion of free protein, indicating that the conjugate was formed in high yield. No high molecular weight protein is evident in the mixture fractions. Only conjugate and essentially no free protein is evident in the conjugate fractions. This confirms that a conjugate formed in high yield.

Example 16: Preparation of BSA-Pn14 Conjugates via Glycidic Acid and Amino-Oxy Derivatized Pn-14.

The following example is illustrative of the preparation of a conjugate using an aldehyde-substituted protein.

I. In situ synthesis of NHS ester of glycidic acid using TSTU and addition to BSA

In this step, 7.9 mg of glycidic acid hemi-calcium salt monohydrate (MW 143) was solubilized in 110 µl NMP. This was combined with 200 µl of 0.5 M TSTU (Novachem) in NMP, and 100 µl of triethylamine, and was added to 1 ml of 24 mg/ml BSA. The pH was adjusted to pH 8. After approximately 2 hours, the mixture was dialyzed on 2 x 1 liter saline. The number of free amines on BSA was determined using TNBS. For the control, the number was 33.2 NH_2/BSA. For glycidic acid/TSTU/BSA, the number was 25 NH_2/BSA. These results lead to the conclusion that BSA was labeled with about 8 glycidic acid units /BSA

II. Oxidation of Functionalized BSA

A 5 mg aliquot was made up with 25 mM NaAc at pH 5, and 25 mM sodium periodate. The reaction was allowed to proceed in the dark at room temperature for 15 minutes, after which a drop of glycerol was added to quench the reaction. The mixture was fractionated S200HR, pool main peak & concentrate.

III. Preparation of BSA(ox)-AO-Pn14 Conjugate

In this step, 444 µl of amino-oxy functionalized Pn14 was combined with 0.4 ml BSA(ox) made up at 10.1 mg/ml, and 100 µl 1 M NaAc at pH 5, and reacted overnight at room temperature. The Fractionate by gel filtration S400HR 1x60 cm saline + 0.02% azide SEC HPLC indicated that oxidation of the Glycdic acid/TSTU/BSA caused polymerization of the BSA.

Also observed was the progressive increase in the high molecular weight peak, indicating that conjugation was increasing with time. The AO-Pn14 alone had minimal absorbance.

Example 17: Preparation of Mercaptoglycerol-Bromoacetate BSA

This example illustrates a process for preparing a BSA(mercaptoglycerol(ox))-AO-dextran conjugate.

Preparation of bromoacetylated BSA

500µl monomeric BSA (48mg/ml) was combined with 500µl 1 M HEPES, at pH 8, and 25 µl 0.1 M NHS bromoacetate in NMP. For the control, 250 µl BSA was combined with 250 µl HEPES and 12 µl NMP

After approximately 1 hour, each was desalted into saline using an Amican Ultra 4 (30 kDa cutoff) device. The final volume was 450 µl BSA-bromoAc, and 300µl BSA control

Next, 50 mM mercaptoethanol and 50 mM mercaptoglycerol were prepared in water.

Preparation E: 225 µl BSA-BromoAc was combined with 100 µl 1 M HEPES at pH 8 and 50 µl of 50 mM mercaptoglycerol.

Preparation F: The BSA control was combined with 100 µl 1 M HEPES to pH 8 and 50 µl 50 mM mercaptoglycerol

Preparation G: 225 µl BSA- BromoAc was combined with 100 µl 1 M HEPES at pH 8, and 50 µl 50 mM mercaptoethanol.

After 30 minutes, each was desalted with Amicon Ultra using NaAc buffer (10 mM NaAcetate, 150 mM NaCl, 5 mM EDTA, pH 5). Final volume was 0.5 ml

Each was then made up in 10 mM sodium periodate from a freshly prepared 0.5 M stock and incubated for 10 minutes at 4°C in the dark, and then quenched by the addition of glycerol and desalted using the Amicon Ultra device and washed into NaAc buffer. By OD 280, each was determined to be about 20 mg/ml BSA.

Preparation E should contain BSA-aldehyde, Preparation F was not labeled with the bromoacetate, and so it could not react with the mercaptoglycerol. Thus, it should not contain aldehydes. Preparation G would have pendent mercaptoethanol, which does not oxidize, so it should not contain aldehdydes.

315 µl of Amino-oxy dextran, at 15.9 mg/ml, was combined with 250 µl of each BSA preparation, and incubated overnight at room temperature in the dark.

Each was then fractionated by gel filtration on a S400HR 1x60cm equilibrated with saline. The high molecular weight fraction was analyzed for protein and dextran.

The results indicated that only BSA containing the oxidized mercaptoglycerol formed a conjugate, and this was confirmed by the protein/dextran ratio of the high molecular weight fraction.

E BSA-mercaptoglycerol(ox)	+ AO-dex	0.97 mg BSA/mg dex.
F BSA control (ox)	+ AO-dex	0.1 mg BSA/mg dex.

G BSA-mercaptoethanol(ox) + AO-dex 0.1 mg BSA/mg dex.

Example 18: Linking of a Protein to a Polysaccharide via Oxime Formation

The following example illustrates the linking of a protein via its N-terminal group to a polysaccharide via oxime formation.

N-terminal threonine of lysostaphin was oxidized and derivatized with a bis-amino-oxy reagent. Oxidation of the protein was performed as generally described in Gaertner & Offord, "Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins," *Bioconjugate Chem.* 7:38 (1996). Lysostaphin a 27 kDa protein was produced in lactococcus.

Trial No. 1

The lysostaphin used contained only about 30% free N-terminal threonine. Conditions of Gaertner & Offord were used for oxidation of the N-terminal threonine. In more detail, a 50 molar excess of methionine (17.5 µl from a 1M stock in water) was added to 1 ml of a 10 mg/ml solution of lysostaphin. Sodium bicarbonate (1M) was added to adjust the pH to 8.3. Oxidation was commenced by the addition of sodium periodate (7 µl from a 0.5 M stock in water). The reaction mixture was kept in the dark at room temperature for 10 minutes, at which time 7.1 mg Bis(amino-oxy)tetraethylene glycol (obtained from Solulink™) prepared as a 50 mg/ml solution in DMSO was added. After 1 hour in the dark, the solution was dialyzed against saline in the dark at room temperature. The product is termed lysotaphin AO. The lysostaphin concentration was determined at OD 280 using 0.49 mg/ml/Absorbance unit.

An aliquot was tested with TNBS at pH 5. It has previously been found that amino-oxy but not amines reacted with TNBS under these conditions. The assay was performed as follows: 50 µl of lysostaphin or lysostaphin AO was added to 440 µl of 0.1 M NaAc, pH 5 and then 10 µl of 10 mg/ml TNBS in water added. 5 µl of 1 mM Amino-oxy acetic acid was used as a standard in the above solution.

Samples were read at 500 nm after a 6 hour incubation in the dark. The sample solution was orange, indicating presence of amino-oxy groups. Using the standard, it was estimated about 30% of lysostaphin was derivatized with the AO group. This lysostaphin AO was then reacted with excess oxidized T2000 dextran in the dark at room temperature to allow conjugation via oxime formation. The reaction was assayed by SEC HPLC to determine the shift of mass from low molecular weight (unconjugated protein) to high molecular weight (lysostaphin-dextran conjugate). A Phenomenex Biosep SEC2000 (300x4.6) equilibrated with PBS and run at 0.5 ml/min with monitoring at 280 nm was used for SEC HPLC

With reference to Figure 6, the upper chromatogram is the reaction mixture at about 1 minute, the middle chromatogram is after an overnight reaction and the lower figure is the lysostaphin AO alone. Note the shift to high molecular weight material after the reaction was allowed to proceed overnight. This figure suggests that the AO group on the lysostaphin linked to the high molecular weight, oxidized dextran. About 27% was coupled, based on the percentage of the area of the high molecular weight peak. This is in the expected percentage since only about a third of the lysostaphin contained a free threonine and was derivatized with AO, as indicated by TNBS assay.

Example 19: Preparation of DT(ox)-AO-Pn14 Conjugate

This example illustrates the preparation of the DT(ox)-AO-Pn14 conjugate, and it also demonstrates how reagents can be prepared in as "single pot" reactions (which may simplify preparation).

I. Mercaptoglycerol-Diptheria toxoid

0.5 ml diphtheria toxoid (~13 mg/ml) was combined with 100 µl 1 M HEPES, pH 8 and 10 µl 0.1 M NHS bromoacetate in NMP. It was incubated in the dark for about 30 minutes, and then 10 µl of 12.3 µl mercaptoglycerol was added.

Following an overnight reaction, the solution was desalted with an Amicon Ultra 4 (30kDa cutoff) to a final volume of about 400 µl.

Next, 50 µl of 1 M sodium acetate at pH 5 was added, followed by 9 µl of 0.5 M sodium periodate. Oxidation was allowed to proceed for 10 minutes in the dark at room temperature. The reaction was then quenched by the addition of 50% glycerol. The low molecular weight components were removed on the same Amicon Ultra 4 device and diafiltered into saline. The final volume was about 200 µl.

The above protocol eliminated one of the desalting steps by adding excess mercaptoglycerol to the solution containing bromoacetylated-DT and bromoacetate.

II. Conjugation

1 ml of amino-oxy Pn14 (~9 mg/ml) was added to the oxidized DT and 100 µl of 1 M sodium acetate at pH 5 added. The reaction was allowed to proceed overnight at room temperature in the dark, and then fractionated on an S400HR column (1x60 cm, equilibrated with saline).

The high molecular weight fraction was pooled. Protein was estimated using 1 mg/ml= 1 OD and the Pn14 concentration determined using the resorcinol assay. The fraction was found to contain about 1.3 mg DT/mg Pn14.

Example 20: Preparation of a gp350(ox)-AO-S-Pn14 conjugate.

gp350 is a glycoprotein from Epstein Barr virus that binds to human complement receptor. It was produced recombinantly in yeast cells by Dr. Goutam Sen (Uniformed Services University of the Health Sciences, Bethsda, MD) and purified by hydrophobic interaction chromatography.

The pH of 0.5 ml of gp350 at 8 mg/ml in PBS was reduced by the addition of 50 µl 1 M sodium acetate, pH 4.7, and 11 µl of 0.5 M sodium periodate (in water) was added. After an 8 minute incubation in the dark, on ice, the reaction